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Identification and Characterization of Components of the Spindle Checkpoint Pathway in Fission Yeast

Sheila Kadura and Shelley Sazer

During anaphase of mitosis, sister chromatids are separated by the mitotic spindle. The spindle assembly checkpoint protects the integrity of the genome by initiating a cell cycle delay if chromosomes are not properly attached to the spindle. Cells lacking a functional spindle checkpoint may gain or lose genetic information, which can cause cell death or predispose cells to cancer. For example, mutations in human Bub1p have been identified in cancer cell lines and altered expression of the *bub1* gene has been shown to correlate with tumor cell proliferation and chromosomal instability. Most human spindle checkpoint components were identified by their similarity to yeast checkpoint proteins that were discovered through genetic screens. Many aspects of spindle checkpoint function are not yet understood, and genetic evidence indicates there are additional checkpoint proteins that have not been identified. By using a genetic screen in fission yeast, this project aims to identify and characterize novel spindle checkpoint components and novel mutant alleles of known spindle checkpoint genes. To date, mutations in three known yeast spindle checkpoint genes have been identified by this screen. A novel bub1 mutant, called bub1-A78V, was selected for further study because it expresses stable Bub1p but is spindle checkpoint defective, and the bub1-A78V mutation occurs inside the Bub1p Mad3-like region, a domain which is conserved from yeast Bub1p to human Bub1p, but the function of this region remains unknown. Our studies indicate that the Mad3-like region of Bub1p is required for correct localization of Bub1p, Bub3p, and Mad3p inside the nucleus in cycling cells and to unattached kinetochores when the spindle checkpoint is activated.

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Table of Contents

INTRODUCTION

The following is the introduction to a manuscript currently in preparation as listed in Reportable Outcomes on page 22. This manuscript pertains to Tasks 1, 2, 3, and 5 of Specific Aim 1 as stated in the approved Statement of Work.

During anaphase of mitosis, the sister chromatids are separated by the dynamic microtubules that make up the mitotic spindle. Failure to achieve equal distribution of chromosomal DNA can lead to genetic loss and lethality. In eukaryotic cells, the spindle checkpoint protects against genetic loss by preventing cells from initiating anaphase until all chromosomes are properly attached to a bipolar mitotic spindle (for review see Wassmann and Benezra, 2001). Two independent screens in *Saccharomyces cerevisiae* identified the first spindle checkpoint genes (*MAD1*, *MAD2*, *MAD3*, *BUB1*, *BUB2*, and *BUB3*) because mutations in these genes cause cells to be sensitive to microtubule disruption (Hoyt et al., 1991; Li and Murray, 1991). *MPS1* was later identified as an essential gene required for both the spindle checkpoint and spindle pole body duplication (Weiss and Winey, 1996).

Homologues of these spindle checkpoint genes have now been identified in the fission yeast, *Schizosaccharomyces pombe*, and in higher eukaryotes (for review see Millband et al., 2002). In budding yeast and fission yeast, the checkpoint proteins are not essential for viability because they are not required during an unperturbed mitosis (for review see Wassmann and Benezra, 2001). However, *S. pombe* bub1Δ mutants and *S. cerevisiae* bub1Δ and mad2-1 mutants exhibit increased chromosome loss and mis-segregation (Bernard et al., 2001; Pangilinan and Spencer, 1996), and spindle checkpoint genes are essential for accurate meiotic division in both yeasts. Specifically, budding yeast Mad1p and Mad2p (Shonn et al., 2000) and fission yeast Mad2p and Bub1p (Bernard et al., 2001) are required to prevent chromosome mis-segregation in meiosis. In higher eukaryotes, the *mad2* and *bub1* spindle checkpoint genes are essential for viability. *Drosophila* bub1 mutants and mice lacking the *mad2* gene die early in development and exhibit chromosome mis-segregation (Basu et al., 1999; Dobles et al., 2000). Loss of spindle checkpoint function has also been implicated in tumorigenesis (for review see Wassmann and Benezra, 2001), and decreased expression of *mad2* promotes lung tumors in mice (Michel et al., 2001).

Contrary to the expectations from early studies (see Wells, 1996), genetic analyses of checkpoint mutants in budding yeast have made it clear that the spindle checkpoint is not a simple, linear genetic pathway. Prior to checkpoint activation, the checkpoint proteins exist in small subcomplexes. When the checkpoint is activated, the compositions of these subcomplexes are altered (for review see Musacchio and Hardwick, 2002) and the Mps1, Mad1, Mad2, Mad3, Bub1, and Bub3 checkpoint proteins are recruited to unattached kinetochores (for review see Cleveland et al., 2003). Mad2p, Mad3p, and Bub3p prevent progression from metaphase to anaphase by binding to the Anaphase Promoting Complex (APC) and inhibiting its ubiquitin ligase activity through the APC effector, Cdc20p/Slp1p (for review see Yu, 2002). APC inhibition stops cell cycle progression by preventing the ubiquitination and subsequent degradation of proteins, such as Pds1p/Cut2p, by the 26S proteasome. Bub2p acts in a separate pathway to prevent cyclin B degradation and exit from mitosis (for review see Gardner and Burke, 2000).

Bub1p is a protein kinase that is required for spindle checkpoint function in mitosis and meiosis. The Bub1p domain structure is conserved from yeast to human and includes a kinase domain, a Bub3p-binding domain, and a Mad3-like region, the function of which is unknown (Hardwick et al., 2000; Taylor et al., 1998; Warren et al., 2002). The Bub1p kinase activity is required for the checkpoint to be completely activated (Yamaguchi et al., 2003), but in vivo targets of this kinase activity have yet to be identified. Bub1p is a phosphoprotein (for review see Vanoosthuyse and Hardwick, 2003). In *S. pombe*, Bub1p is phosphorylated by Cdc2p, and mutation of the Cdc2p phosphorylation sites prevents the checkpoint from being fully functional (Yamaguchi et al., 2003). Bub1p localizes to mitotic kinetochores in every cell cycle (Bernard et al., 1998; Toyoda et al., 2002) and is required for localization of Mad1p, Mad2p, Mad3p, and Bub3p to unattached kinetochores during the checkpoint response (Millband and Hardwick, 2002; Sharp-Baker and Chen, 2001). Bub1p localization to kinetochores requires Mad3p and Bub3p (Chen, 2002; Taylor et al., 1998). Bub1p binds to Bub3p throughout the cell cycle (for review see Millband et al., 2002) and to Mad1p and Bub3p during mitosis (Brady and Hardwick, 2000). The Bub1p-Mad1p-Bub3p complex is stabilized in checkpoint-activated cells and is required for checkpoint function.

Previous work in our lab identified the first two spindle checkpoint genes in *S. pombe*: *mad2* and *mph1* (He et al., 1998; He et al., 1997). Deletion of *mad2* or *mph1* renders cells sensitive to the microtubule-destabilizing drug, thiabendazole (TBZ), and overexpression of either *mad2* or *mph1* activates the spindle checkpoint, arrests cells prior to anaphase, and is toxic to wild type cells. In the spindle checkpoint pathway, *mph1* acts upstream of *mad2*. Therefore, while *mph1* overexpression arrests the cell cycle in wild type cells, this toxic effect is not observed in mad2Δ mutants that lack a functional checkpoint.

To identify novel checkpoint genes and novel mutations in previously identified checkpoint genes, a genetic screen was conducted to isolate strains that, like the mad2Δ strain, can survive *mph1* overexpression. To date, this screen has identified five novel mutant alleles of three checkpoint genes: bub1-R988Stop, bub1-A78V, bub1-G139D, bub3-G255D, and mad1-Q386Stop. We describe in detail the Bub1p mutant, bub1-A78V, which is mutated in the Mad3-like region, a domain that is conserved from yeast Bub1p to human Bub1p (Hardwick et al., 2000; Taylor et al., 1998; Warren et al., 2002) but has not been previously functionally examined. Our studies indicate that the Mad3-like region of Bub1p is essential for accumulation of Bub1p, Bub3p, and Mad3p inside the nucleus and for localization of these proteins to unattached kinetochores when the checkpoint is activated.

Results

The following is the results section of a manuscript currently in preparation. Figures and figure legends can be found following this section on pages 10-16.

The *mph1* overexpression screen identified five new alleles of known spindle checkpoint genes.

In *S. pombe*, *mph1* overexpression activates the spindle checkpoint and arrests cells in mitosis (He et al., 1998). This effect of *mph1* overexpression is not observed in mad2Δ cells, indicating that *mad2* acts downstream of *mph1* and that the ability to survive *mph1* overexpression can be used as a criterion for identifying mutants in checkpoint genes that act downstream of *mph1*. This concept was used to design a screen to identify novel spindle checkpoint genes and novel mutants of previously identified checkpoint genes in *S. pombe*.

Chemically mutagenized strains were tested for ability to survive overexpression of *mph1* as the primary criterion for the genetic screen. As a secondary test, strains were tested for sensitivity to the microtubule-destabilizing drug, thiabendazole (TBZ), a characteristic of strains with defects in microtubules and/or the spindle checkpoint. Of 250 strains tested, 183 were TBZ-sensitive. 16 of these strains were chosen for further study based on their strong sensitivity to TBZ and normal growth in the absence of TBZ.

To determine if these 16 strains are mutated in a previously identified spindle checkpoint gene, each strain was crossed to mad1Δ, mad2Δ, mad3Δ, bub1Δ, and bub3Δ strains (K. Hardwick, unpublished data, Bernard et al., 1998; He et al., 1998; He et al., 1997; Millband and Hardwick, 2002). 5 of the 16 strains carry a mutation that is tightly linked to a known checkpoint gene. PCR amplification of the mutant alleles and nucleotide sequencing of their open reading frames confirmed that three *bub1* alleles, one *bub3* allele and one *mad1* allele were identified in the screen (see Figure 1).

The three identified bub1 mutations occur in regions of *S. pombe* Bub1p that are similar to the Mad3-like domain and protein kinase domain of *S. cerevisiae* Bub1p (Figure 1A). The bub1-R988Stop mutation introduces a premature stop codon in the protein kinase domain which removes 82 amino acids from the carboxy terminus of Bub1p. The bub1-A78V and bub1-G139D mutations introduce single amino acid changes in the conserved Mad3-like region. In addition, we identified a mutation in Bub3p, bub3-G255D, which alters a residue that is conserved in the budding yeast and human Bub1 proteins and is located in a predicted WD40 domain of fission yeast Bub3p (Figure 1B). We also identified a mutation, mad1-Q386Stop, which introduces a premature stop codon in a coiled-coil region of Mad1p, removing 304 amino acids from the carboxy terminus of the protein (Figure 1C). All five of the isolated mutant alleles exhibit a sensitivity to TBZ similar to their respective null strains (data not shown) indicating that these alleles are loss of function mutations. In this study, we describe further analysis of the three novel bub1 mutants identified in this screen.

The bub1-A78V mutant is defective in the spindle checkpoint and expresses stable Bub1p.

A Western blot was performed to ascertain whether these mutant genes encode stable Bub1p. bub1-A78V, but not bub1-R988Stop or bub1-G139D, expresses Bub1p at the wild type level (Figure 2A). When the spindle checkpoint is activated by *mph1* overexpression in cells that express GFP-tubulin to allow visualization of microtubules (Ding et al., 1998), wild type cells exhibit short spindles, indicating that the cell cycle is arrested at the metaphase to anaphase transition (Figure 2B). In contrast, bub1-A78V cells do not accumulate short spindles during *mph1* overexpression, illustrating that they do not have an intact spindle checkpoint. bub1-A78V cells are as sensitive to microtubule disruption as a bub1Δ strain, indicating that bub1-A78V is as checkpoint defective as the bub1Δ (Figure 2C). These results indicate that the Bub1p Mad3-like region is required for the spindle checkpoint, and since bub1-A78V expresses stable Bub1p, this strain can be used to further characterize the role of the Mad3-like region in checkpoint function.

The Bub1p, Mad3p, and Bub3p spindle checkpoint proteins are mis-localized in the bub1-A78V mutant.

S. pombe Bub1p localizes predominantly to the nucleus in interphase cells. To begin characterizing the checkpoint defect in the bub1-A78V mutant strain, we first determined whether the A78V mutation disrupts the nuclear localization of Bub1p. We constructed a Bub1-A78V-GFP strain and compared localization of the mutant protein to wild type Bub1-GFP in cycling cells. While Bub1-GFP accumulates inside of the nucleus and co-localizes with DNA, Bub1-A78V-GFP is diffusely localized throughout the cell (Figure 3A).

Physical interactions have been reported between Bub1p, Bub3p, and Mad1p, and between Bub3p and Mad3p (for review see Millband and Hardwick, 2002). Therefore, we wondered whether mis-localization of Bub1p in the bub1-A78V mutant would disrupt the localization of these checkpoint proteins in cycling cells. Mad1-GFP localizes to the nucleus in a punctate pattern that is indicative of Mad1p's association with nuclear pore complexes in wild type cells (Figure 3B), and this localization is unaltered in bub1-A78V or bub1Δ cells. Bub3-GFP and Mad3-GFP localize predominantly to the nucleus in wild type cells (Figure 3C and 3D). However, Bub3-GFP and Mad3-GFP are diffusely localized throughout the bub1-A78V cells (Figure 3C and 3D). This pattern of mis-localization is similar to what is observed in the bub1Δ strain (Figure 3C and 3D). These results indicate that the Mad3-like region of Bub1p is required for correct localization of Bub1p, Bub3p, and Mad3p inside the nucleus in cycling cells.

Bub1-A78V-GFP does not localize to kinetochores during a normal mitosis or when the spindle checkpoint is activated.

Bub1p localizes at the kinetochore during every cell cycle and persists at the kinetochore during checkpoint activation (Bernard et al., 1998; Toyoda et al., 2002). It is possible that the bub1-A78V mutant is spindle checkpoint defective because Bub1p can not be recruited to kinetochores in these cells. To test this hypothesis, we compared

Bub1-GFP and Bub1-A78V-GFP localization in cycling cells grown in rich medium (YE) and cells that were synchronized in S phase by hydroxyurea (HU) to enrich for mitotic cells upon release into YE or YE containing the microtubule-destabilizing drug, carbendazim (CBZ), which activates the spindle checkpoint.

In cycling cells or cells synchronized in S phase before release into YE, Bub1-GFP is predominantly localized in the nucleus while Bub1-A78V-GFP is diffusely distributed throughout the cell (Figure 4A). Bub1-GFP localizes to kinetochores in mitotic cells (Figure 4A), while Bub1-A78V-GFP is not observed at kinetochores as illustrated by the lack of a kinetochore GFP signal in binucleate anaphase bub1-A78V cells. When the checkpoint is activated by treatment with CBZ for 2 hours, many wild type cells exhibit bright Bub1-GFP kinetochore dots. This kinetochore localization is not observed in Bub1-A78V-GFP cells. This result is not a consequence of the spindle checkpoint defect in bub1-A78V cells because these cells can recruit Mad1-GFP to kinetochores when the checkpoint is activated by CBZ (see Figure 5). Further, there is no difference in the number of mitotic cells in the bub1-A78V strain when compared to a wild type strain in the presence of CBZ at this time point (data not shown).

Rarely, Bub1-A78V-GFP was observed at kinetochores in mitotic or checkpoint-activated cells, but the GFP signal in these cells was much less robust than Bub1-GFP kinetochore localization (Figure 4B). A Western blot confirmed that the observed mis-localization of Bub1-A78V-GFP was not due to decreased stability of Bub1-A78V-GFP compared to Bub1-GFP (Figure 4C). These results indicate that the Mad3-like region of Bub1p is required for kinetochore localization of Bub1p.

Bub3-GFP and Mad3-GFP do not localize to kinetochores when the spindle checkpoint is activated in bub1-A78V cells.

Bub1p binds to Bub3p constitutively in yeast and mammals (for review see Millband et al., 2002) and associates with Mad1p and Bub3p when the spindle checkpoint is activated in budding yeast (Brady and Hardwick, 2000). Mad1p, Bub3p, and Mad3p are recruited to unattached kinetochores when the spindle checkpoint is activated (for review see Cleveland et al., 2003). Experiments in *Xenopus laevis* have demonstrated that recruitment of Mad1p, Mad2p, and Bub3p to unattached kinetochores requires Bub1p (Sharp-Baker and Chen, 2001) while studies in *S. pombe* have shown that Mad3p localization to unattached kinetochores is dependent on Bub1p (Millband and Hardwick, 2002). In light of these findings, we wanted to determine whether Mad1-GFP, Bub3-GFP, and Mad3-GFP can localize to kinetochores when the checkpoint is activated in bub1-A78V cells.

In cycling cells or cells synchronized in S phase by HU prior to release into YE, Mad1-GFP localizes inside the nucleus in wild type, bub1-A78V, and bub1Δ cells (Figure 5A). When the checkpoint is activated by CBZ, Mad1-GFP localizes to kinetochores in wild type cells. This kinetochore localization is also observed in bub1-A78V and bub1Δ cells (Figure 5A). In contrast, neither Bub3-GFP nor Mad3-GFP localizes to kinetochores when the checkpoint is activated in the bub1-A78V mutant (Figure 5B and 5C). Similar results were observed for the bub1Δ strain (Figure 5B and 5C). A Western blot was performed to confirm that Bub1p-A78V is equivalently produced in the Mad1-GFP, Bub3-GFP, and Mad3-GFP strains (Figure 5D).

Figure Legends

Figure 1. The *mph1* overexpression identified five mutant alleles of three checkpoint genes. (A) Comparison of the known domains in *S. cerevisiae* Bub1p and similar domains in *S. pombe* Bub1p. The bub1-A78V and bub1-G139D mutations occur in the conserved Mad3-like region. The bub1-R988Stop mutation introduces a premature stop codon in the protein kinase domain which removes 82 amino acids from the carboxy terminus of Bub1p. (B) *S. pombe* Bub3p contains three WD40 domains. The bub3-G255D mutation occurs in the third predicted WD40 domain. (C) *S. pombe* Mad1p contains five predicted coiled-coil domains. The mad1-Q386Stop mutation introduces a premature stop in the third coiled-coil region removing 304 amino acids. Red arrows indicate mutations identified in this study.

Figure 2. The bub1-A78V strain expresses stable Bub1p but can not arrest the cell cycle in response to *mph1* overexpression and is sensitive to TBZ. (A) A Western blot using an anti-Bub1p polyclonal antibody to compare Bub1p levels in the bub1 point mutants to wild type and bub1Δ cells. The bub1-A78V strain, but not the bub1-R988Stop or bub1-G139D strains, expresses the wild type level of Bub1p. (B) *mph1* was overexpressed in wild type and bub1-A78V cells expressing GFP-tubulin to visualize microtubules. The presence of short mitotic spindles in wild type cells overexpressing *mph1* indicates an arrest at the metaphase to anaphase transition. bub1-A78V cells with short spindles do not accumulate under these conditions. (C) Cells were grown in rich medium and spotted onto plates in five fold dilutions in the presence or absence of TBZ. The bub1-A78V strain is as sensitive to TBZ as the bub1Δ strain.

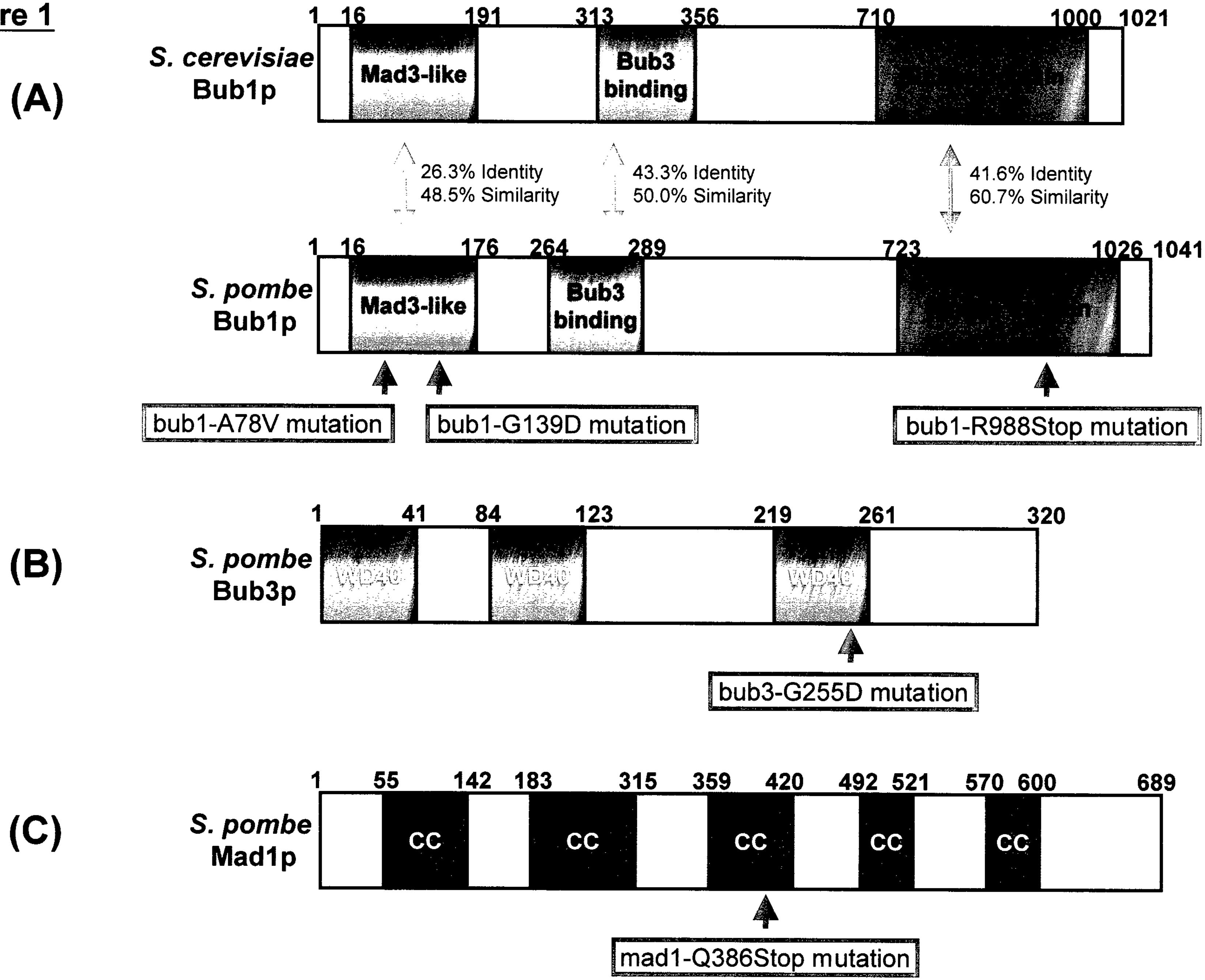
Figure 3. Bub1-GFP, Bub3-GFP, and Mad3-GFP are mis-localized in the bub1-A78V mutant. (A) Strains expressing C terminally GFP-tagged Bub1 or Bub1-A78V driven by the native *bub1* promoter at its endogenous locus were cultured in rich medium (YE). Live cells were examined to observe localization of the GFP-tagged protein, and DNA was detected by staining with Hoechst 33342. (B-D) Strains expressing C terminally GFP-tagged Mad1, Bub3, or Mad3 driven by their native promoters at their endogenous loci were cultured and observed as in (A). (B) Wild type, bub1-A78V,and bub1Δ cells expressing Mad1-GFP. (C) Wild type, bub1-A78V,and bub1Δ cells expressing Bub3-GFP. (D) Wild type, bub1-A78V,and bub1Δ cells expressing Mad1-GFP.

Figure 4. Bub1-A78V-GFP does not localize correctly to the nucleus during interphase or to kinetochores during mitosis or checkpoint activation. (A) Cells expressing C terminally tagged Bub1-GFP or Bub1-A78V-GFP driven by the native *bub1* promoter at its genomic locus were cultured in rich medium (YE) or synchronized in S phase by hydroxyurea to enrich for mitotic cells upon release into YE (YE+HU) or into YE with carbendazim (YE+CBZ), which activates the spindle checkpoint. Kinetochore localization of wild type Bub1p (arrows) occurs during a normal mitosis (asterix) or when the spindle checkpoint is activated by CBZ. (B) Bub1-GFP and Bub1-

A78V-GFP expressing cells were synchronized in HU and released into YE with CBZ. Bub1-A78V-GFP rarely localizes to kinetochores (arrow) during checkpoint activation, and this localization is less robust than is observed for Bub1-GFP. (C) Western blot using an anti-Bub1p polyclonal antibody to determine the level of Bub1-GFP and Bub1-A78V-GFP.

Figure 5. Bub3-GFP and Mad3-GFP do not localize to kinetochores when the spindle checkpoint is activated in bub1-A78V cells. Wild type, bub1-A78V, and bub1Δ cells expressing C terminally GFP-tagged Mad1, Bub3, or Mad3 driven by their native promoters at their endogenous loci were cultured in rich medium (YE) or synchronized in S phase by hydroxyurea to enrich for mitotic cells upon release into YE (YE+HU) or into YE with carbendazim (YE+CBZ), which activates the spindle checkpoint. GFP localization was observed in live cells. Arrows denote kinetochore localization of the GFP-tagged protein. (A) Wild type, bub1-A78V and bub1Δ cells expressing Mad1-GFP. (B) Wild type, bub1-A78V, and bub1Δ cells expressing Bub3-GFP. (C) Wild type, bub1-A78V, and bub1Δ cells expressing Mad3-GFP. (D) Western blot using an anti-Bub1p polyclonal antibody to compare the level of Bub1p-A78V in the strains examined in panels A-C.

Figure 1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1
16
191
313
356
710
1000
1021
Mad3-like
Bub3 binding
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48.5% Similarity
43.3% Identity
50.0% Similarity
41.6% Identity
60.7% Similarity
S. pombe Bub1p
1
16
176
264
289
723
1026
1041
Mad3-like
Bub3 binding
bub1-A78V mutation
bub1-G139D mutation
bub1-R988Stop mutation
(B)
S. pombe Bub3p
1
41
84
123
219
261
320
WD40
WD40
WD40
bub3-G255D mutation
(C)
S. pombe Mad1p
1
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CC
CC
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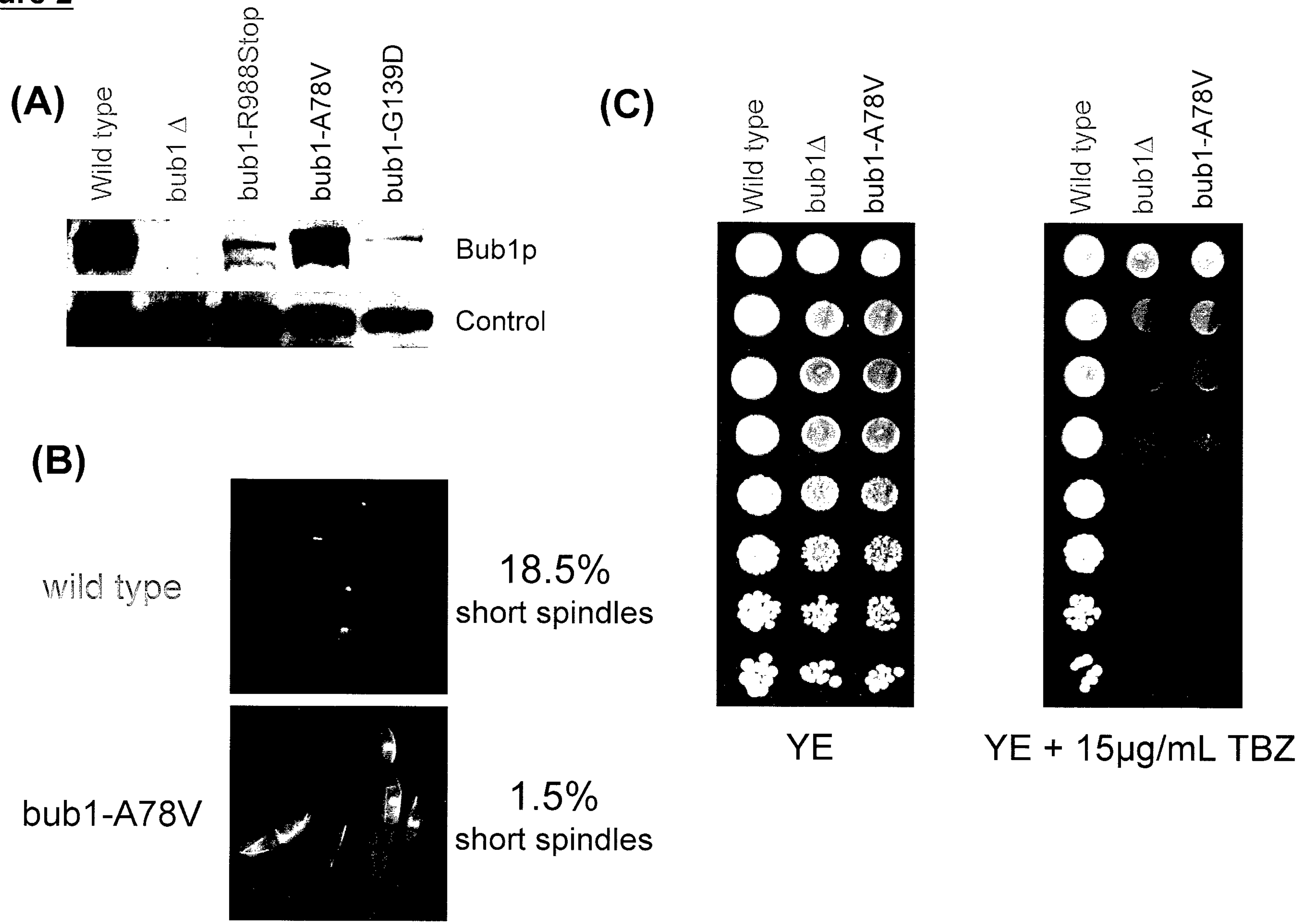
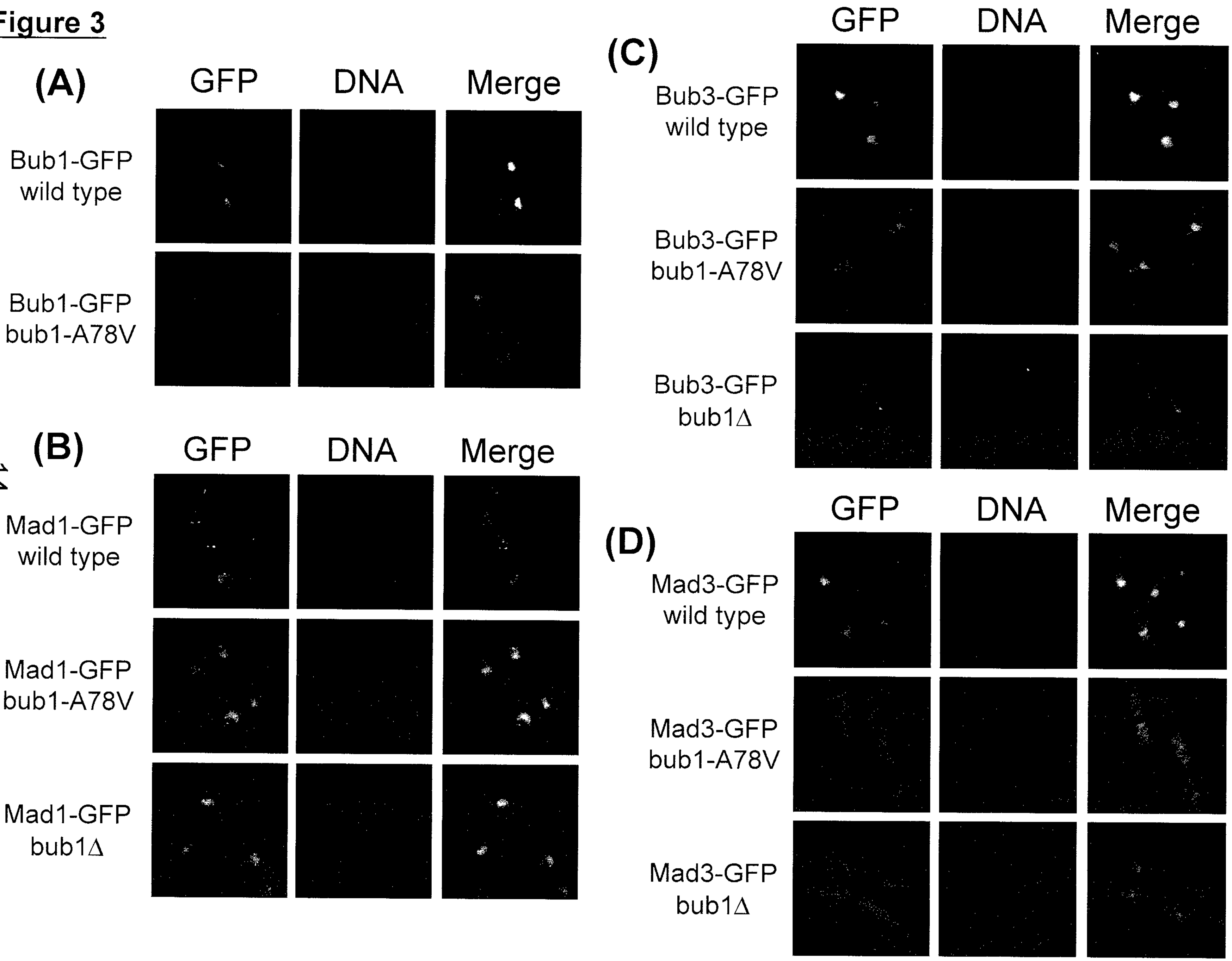
CC
CC
mad1-Q386Stop mutation

Figure 2



(A)
Wild type
bub1 Δ
bub1-R988Stop
bub1-A78V
bub1-G139D
Bub1p
Control
(B)
wild type
18.5%
short spindles
bub1-A78V
1.5%
short spindles
(C)
Wild type
bub1Δ
bub1-A78V
YE
Wild type
bub1Δ
bub1-A78V
YE + 15μg/mL TBZ

Figure 3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Mad1-GFP bub1Δ
(C)
GFP
DNA
Merge
Bub3-GFP wild type
Bub3-GFP bub1-A78V
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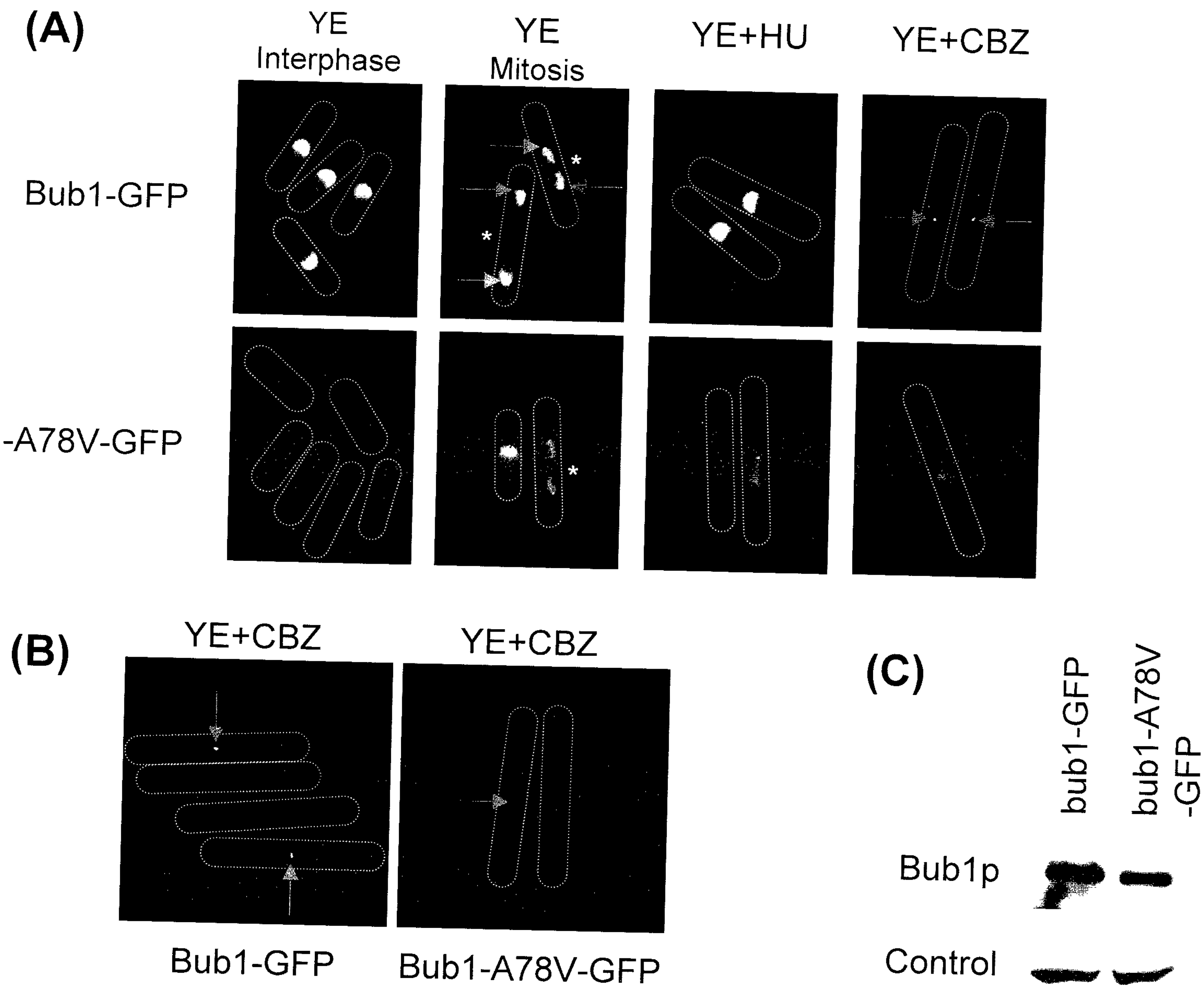
Mad3-GFP bub1Δ

Figure 4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YE Mitosis
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YE+CBZ
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(B)
YE+CBZ
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(C)
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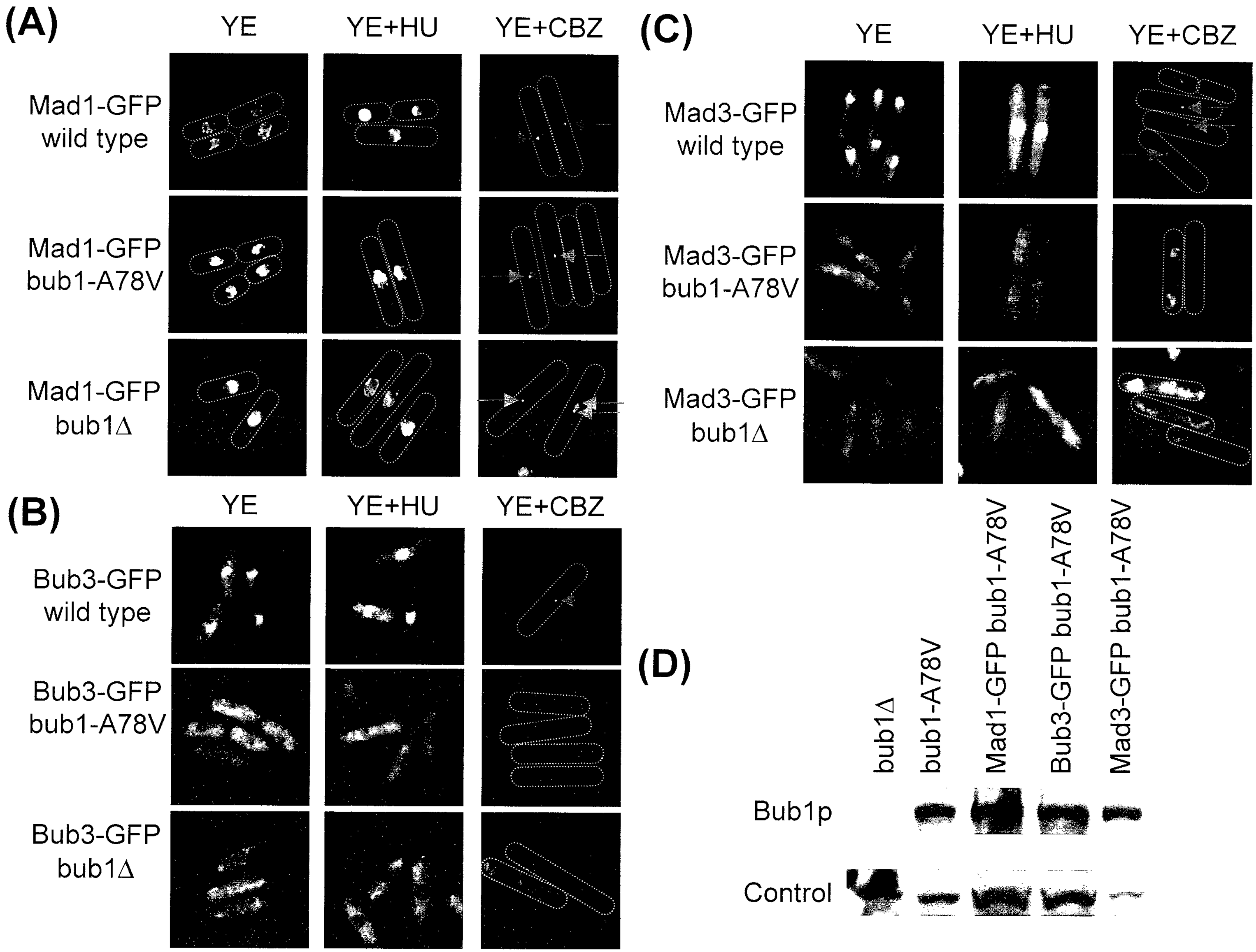
bub1-A78V-GFP
Bub1p
Control

Figure 5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YE+HU
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Mad1-GFP wild type
Mad1-GFP bub1-A78V
Mad1-GFP bub1Δ
(B)
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YE+CBZ
Bub3-GFP wild type
Bub3-GFP bub1-A78V
Bub3-GFP bub1Δ
(C)
YE
YE+HU
YE+CBZ
Mad3-GFP wild type
Mad3-GFP bub1-A78V
Mad3-GFP bub1Δ
(D)
bub1Δ
bub1-A78V
Mad1-GFP bub1-A78V
Bub3-GFP bub1-A78V
Mad3-GFP bub1-A78V
Bub1p
Control

Discussion

The *mph1* overexpression screen was conducted to isolate novel spindle checkpoint genes and novel mutations in known checkpoint genes. This screen has succeeded in identifying five novel mutant alleles of three previously identified spindle checkpoint genes: bub1-R988Stop, bub1-A78V, bub1-G139D, bub3-G255D, and mad1-Q386Stop. The work presented here focuses on the bub1 mutants. In addition, 11 strains have been identified which survive *mph1* overexpression and are sensitive to TBZ but are not mutated in a known spindle checkpoint gene, indicating that these strains may contain mutations in novel checkpoint genes. Also, the *bub3* gene in bub3-G255D is predicted to encode a protein that is mutated in a highly conserved glycine residue which is located in the third predicted WD40 repeat domain. Bub3p has been isolated in spindle checkpoint protein complexes which include Mad1p, Mad2p, Mad3p, Cdc20p, and Bub1p (Brady and Hardwick, 2000; Fraschini et al., 2001; Roberts et al., 1994), and disruption of the third WD40 domain in *S. pombe* Bub3p may disrupt one or more of these interactions. The *mad1* gene in mad1-Q386Stop is predicted to encode a protein that is truncated in the third coiled-coil region, removing 304 amino acids from the carboxy terminus of the protein including a region shown to be required for the formation of a Mad1p-Bub1p-Bub3p complex that becomes stabilized when the checkpoint is activated in *S. cerevisiae* (Brady and Hardwick, 2000).

Of the three novel bub1 alleles identified in this study, only bub1-A78V expresses stable Bub1p. The bub1-A78V strain is mutated in the Mad3-like region of Bub1p, a region that is evolutionarily conserved in Bub1 proteins (Hardwick et al., 2000; Taylor et al., 1998; Warren et al., 2002), but its functional importance is not known. The bub1-A78V strain is sensitive to TBZ and is defective in the spindle checkpoint. Therefore, by further characterizing the checkpoint defect in the bub1-A78V mutant, we can investigate the importance of the Mad3-like region in checkpoint function.

Compared to the localization of Bub1-GFP, Bub1-A78V-GFP fails to accumulate inside the nucleus in interphase cells and does not localize to kinetochores during mitosis or when the spindle checkpoint is activated. These results indicate that a mutation in the Mad3-like region of Bub1p prevents correct localization of Bub1p in interphase, mitotic, and checkpoint-activated cells. The inability of Bub1-A78V-GFP to localize to kinetochores may be related to the decreased nuclear accumulation of Bub1-A78V-GFP. It is possible that the Bub1-A78V-GFP that is inside the nucleus can associate with kinetochores, but this quantity of GFP is not detectable by our methods. There were rare instances of Bub1-A78V-GFP localization to kinetochores, but this localization was much less robust than Bub1-GFP kinetochore localization. The decreased localization of Bub1p to unattached kinetochores could explain the checkpoint deficiency observed in bub1-A78V cells. Petersen and Hagan reported that fission yeast cells lacking Ark1p recruit less Mad2p to unattached kinetochores than wild type cells when the checkpoint is activated by CBZ (Petersen and Hagan, 2003). The authors hypothesize that Ark1p-deficient cells are sensitive to CBZ because decreased localization of Mad2p to unattached kinetochores prevents downstream binding interactions that are required for the spindle checkpoint to arrest the cell cycle.

Mad1-GFP was able to localize correctly in bub1-A78V cells and bub1Δ cells, indicating that Bub1p is not required for correct localization of Mad1p in *S. pombe* although experiments performed in *Xenopus laevis* have shown that Bub1p is required for Mad1p recruitment to unattached kinetochores (Sharp-Baker and Chen, 2001). In *S. cerevisiae*, a Mad1p-Bub1p-Bub3p complex forms during mitosis and is required for checkpoint function (Brady and Hardwick, 2000). It is not known if this complex exists in *S. pombe*, but it is clear that a Mad1p-Bub1p-Bub3p complex is not required for Mad1p localization in *S. pombe*.

We observed that Bub3-GFP is not recruited to unattached kinetochores in checkpoint-activated bub1Δ cells. This result is consistent with experiments in higher eukaryotes which have shown that Bub3p localization to kinetochores is dependent on Bub1p (Basu et al., 1998; Sharp-Baker and Chen, 2001). Likewise, Mad3-GFP does not localize to kinetochores when the checkpoint is activated in bub1Δ cells, which is consistent with previously published experiments in fission yeast (Millband and Hardwick, 2002). We now show that disruption of the Mad3-like region of *S. pombe* Bub1p is sufficient to cause mis-localization of Bub3p and Mad3p in interphase and checkpoint-activated cells. Bub1p-A78V may be unable to recruit Bub3p or Mad3p to kinetochores because the mutation disrupts a critical protein-binding domain, or because it prevents the normal accumulation of Bub1p-A78V inside the nucleus.

hBub1p is phosphorylated in the presence of the microtubule poisons, nocodazole or taxol (Taylor et al., 2001), and phosphorylation of *S. pombe* Bub1p by Cdc2p affects spindle checkpoint function (Yamaguchi et al., 2003). We do not believe that the bub1-A78V mutation alters the phosphorylation state of Bub1p because this mutation does not occur in one of the Cdc2p consensus sites reported in Yamaguchi et al., and we did not observe any difference in migration between wild type Bub1p and Bub1p-A78V in an SDS gel, although we have not yet analyzed these proteins in spindle checkpoint-activated cells.

The Mad3-like region in *S. cerevisiae* Mad3p has been functionally examined and shown to mediate binding between Mad3p and the APC effector protein, Cdc20p (Hardwick et al., 2000). Mad3p and Cdc20p are part of a complex that also includes Mad2p and Bub3p and functions to inhibit APC ubiquitin ligase activity and prevent cell cycle progression from metaphase to anaphase (for review see Yu, 2002). There is no evidence that Bub1p is involved in this complex, and our results indicate that the Mad3-like region of Bub1p is involved in localizing spindle checkpoint proteins inside the nucleus and to unattached kinetochores when the checkpoint is activated.

Bub1p constitutively binds to Bub3p in yeast and higher eukaryotes (for review see Millband et al., 2002) The Bub3p-binding domain of Bub1p has been mapped and does not include the conserved Mad3-like region of Bub1p. We do not yet know if Bub1p-A78V retains its ability to bind to Bub3p, and experiments are currently in progress to answer this question. Determining whether there is a physical interaction between

Bub1p-A78V and Bub3p, will allow us to distinguish between two possible models consistent with our results

Model 1

If Bub1p retains its ability to bind to Bub3p, we propose the following. Mis-localization of Bub1p-A78V, perhaps due to the bub1-A78V mutation interfering with Bub1p transport into and/or retention inside the nucleus, could cause mis-localization of Bub3p throughout the cell cycle due to the constitutive binding of these two proteins. This idea is consistent with our observations in bub1-A78V cells. This model then predicts that the Mad3-like region of Bub1p functions primarily to localize Bub1p and Bub3p inside the nucleus, where they are required for checkpoint function. Because Bub3p constitutively binds to Mad3p (for review see Millband et al., 2002), failure of Bub3p to accumulate in the nucleus could cause a failure of Mad3p to localize to the nucleus as well. In the bub1-A78V cells, some Bub3p and Mad3p is present inside the nucleus, but perhaps this is too little protein to support kinetochore localization and checkpoint function of these two proteins. In addition, human Bub3p is required for Bub1p localization to kinetochores (Taylor et al., 1998), and depletion of *Xenopus* Mad3p greatly reduces Bub1p kinetochore localization (Chen, 2002). The decreased nuclear accumulation of Bub3p and Mad3p in bub1-A78V cells could decrease the ability of Bub1p to localize to kinetochores.

Model 2

Bub1p and Bub3p are each required for the recruitment of the other to unattached kinetochores (Basu et al., 1998; Sharp-Baker and Chen, 2001; Taylor et al., 1998). If the bub1-A78V mutation prevents Bub1p from binding to Bub3p, neither Bub1p nor Bub3p would be recruited to unattached kinetochores. This prediction is consistent with our bub1-A78V experiments. This in turn would affect Mad3p localization because Mad3p requires Bub1p for its recruitment to unattached kinetochores (Millband and Hardwick, 2002). Since we have also observed that the bub1-A78V mutation disrupts Bub1p, Bub3p, and Mad3p localization to the nucleus, this model would predict that the interaction between Bub1p and Bub3p via the Mad3-like region of Bub1p is required for Bub1p, Bub3p, and Mad3p to accumulate inside the nucleus and to localize to unattached kinetochores.

Key Research Accomplishments

- Five strains that do not arrest the cell cycle in response to *mph1* overexpression are mutated in a known spindle checkpoint gene. The bub1-R988Stop mutation introduces a premature stop codon in the protein kinase domain which removes 82 amino acids from the carboxy terminus of Bub1p. The bub1-A78V and bub1-G139D mutations introduce single amino acid changes in the conserved Mad3-like region. A mutation identified in Bub3p, bub3-G255D, alters a conserved residue and is located in a predicted WD40 domain of fission yeast Bub3p. The mad1-Q386Stop mutation introduces a premature stop codon in a coiled-coil region of Mad1p, removing 304 amino acids from the carboxy terminus of the protein.

- All five of the isolated mutant alleles exhibit a similar sensitivity to TBZ as their respective null strains indicating that these alleles are loss of function mutations.

- All three isolated bub1 mutant alleles contain mutations in regions of Bub1p that are conserved from yeast to human. However, bub1-A78V, but not bub1-R988Stop or bub1-G139D, expresses Bub1p at the wild type level, indicating that bub1-A78V is a useful tool in characterizing the importance of the Mad3-like region in checkpoint function.

- bub1-A78V cells do not arrest at metaphase when the spindle checkpoint is activated by *mph1* overexpression and is as sensitive to microtubule disruption by TBZ as a bub1Δ strain, indicating that the checkpoint defects in these two strains are similar.

- Bub1-GFP is predominantly localized inside the nucleus while Bub1-A78V-GFP is diffusely distributed throughout the cell. In mitotic cells and checkpoint-activated cells, Bub1-GFP localizes to kinetochores while Bub1-A78V-GFP is rarely observed at kinetochores, and this kinetochore localization is much less robust than that of Bub1-GFP. A Western blot confirmed that the observed mis-localization of Bub1-A78V-GFP was not due to decreased stability of Bub1-A78V-GFP compared to Bub1-GFP. These results indicate that the Mad3-like region of Bub1p is required for kinetochore localization of Bub1p.

- Mad1-GFP localizes inside the nucleus in cycling cells and to unattached kinetochores when the checkpoint is activated by treatment with the microtubule-destabilizing drug, CBZ, in wild type, bub1-A78V, and bub1Δ cells. These results demonstrate that correct Mad1-GFP localization does not depend on Bub1p.

- Bub3-GFP is predominantly localized inside the nucleus in wild type cells, while bub1-A78V and bub1Δ cells do not exhibit this nuclear localization. While wild type cells localize Bub3-GFP to kinetochores when the checkpoint is activated by CBZ, bub1-A78V and bub1Δ cells do not. A Western blot confirmed that the observed mis-localization of Bub3-GFP was not caused by decreased production of Bub1p-A78V in the Bub3-GFP background. These results indicate that the Mad3-like region of Bub1p is required for correct localization of Bub3p.

- In wild type cells, but not bub1-A78V or bub1Δ cells, Mad3-GFP accumulates in the nucleus . Wild type cells recruit Mad3-GFP to kinetochores when the checkpoint is activated by CBZ while bub1-A78V and bub1Δ cells do not . A Western blot confirmed that the observed mis-localization was not caused by decreased production of Bub1p-A78V in the Mad3-GFP strain. These results indicate that the Mad3-like region of Bub1p is required for correct localization of Mad3p.

- The goals of Specific Aim 2, to identify the functional regions of the Mad2 protein, have been achieved by others. Therefore, I asked permission not to perform these experiments, and this aim was canceled upon review of my second annual report

Reportable Outcomes

Papers:

Kadura, S., Hardwick, K., and Sazer, S. A novel bub1 mutant reveals that the conserved Mad3-like region of Bub1p is required for correct localization of the Bub1p, Bub3p, and Mad3p checkpoint proteins. In preparation.

Poster Presentations at Scientific Meetings:

Kadura, S., He, X., and Sazer, S. 2002. The spindle assembly checkpoint in fission yeast. Department of Defense Breast Cancer Meeting. Orlando, Florida.

Kadura, S., He, X., and Sazer, S. 2000. The spindle assembly checkpoint in fission yeast. FASEB Yeast Chromosome Structure Meeting. Snowmass Village, Colorado.

Kadura, S., He, X., and Sazer, S. 1999. The spindle assembly checkpoint in fission yeast. Lost Pines Conference. Smithville, Texas.

Poster Presentations at Baylor College of Medicine:

Baylor College of Medicine Graduate Student Symposium – 2000, 2001, 2002.

Molecular and Cellular Biology Departmental Retreat – 2001, 2002, 2003.

Biochemistry and Molecular Biology Departmental Retreat, BCM – 2000, 2001.

Oral Presentations:

Biochemistry and Molecular Biology Departmental Retreat, BCM – 2002, 2003.

Honors

2003	Department of Molecular and Cellular Biology Outstanding Poster Award – First Place, Baylor College of Medicine
2001	Department of Molecular and Cellular Biology Outstanding Poster Award finalist, Baylor College of Medicine.

Education:

I anticipate completing my graduate studies at Baylor College of Medicine in 2004.

Conclusions

To date, the *mph1* overexpression screen has identified five novel mutant alleles of three checkpoint genes: bub1-R988Stop, bub1-A78V, bub1-G139D, bub3-G255D, and mad1-Q386Stop. The Bub1p mutant, bub1A78V, is mutated in the Mad3-like region, a domain that is conserved from yeast Bub1p to human Bub1p (Hardwick et al., 2000; Taylor et al., 1998; Warren et al., 2002) but has not been previously functionally examined. The bub1-A78V strain produces stable Bub1p but is defective in the spindle checkpoint.

I have further characterized the checkpoint defect in the bub1-A78V strain to better understand the function of the conserved Mad3-like region. My studies indicate that the Mad3-like region of Bub1p is essential for accumulation of Bub1p, Bub3p, and Mad3p inside the nucleus and for localization of these proteins to unattached kinetochores when the checkpoint is activated.

Loss of spindle checkpoint function can cause cells to gain or lose genetic information, which can cause cell death or predispose cells to cancer. In support of this prediction, mutations in human Bub1p have been identified in cancer cell lines and altered expression of the *bub1* gene has been shown to correlate with tumor cell proliferation and chromosomal instability (Cahill et al., 1998; Grabsch et al., 2003; Musio et al., 2003; Ru et al., 2002).By studying the mitotic functions of Bub1p, we can better understand how misregulation of Bub1p may promote tumorigenesis.

During my fellowship, I have been able to complete most of the tasks outlined in Specific Aim 1. Specific Aim 2 was cancelled last year because the goals of this aim, to identify the functional regions of the Mad2 protein, have been achieved by others. The goal of Specific Aim 3 is to identify mammalian spindle checkpoint proteins by their ability to arrest the cell cycle when overproduced in *S. pombe*. I have initiated work on this aim. I have refined the conditions for performing this screen by showing that overexpression of *S. pombe mad2* or *mph1* in cells expressing GFP–α-tubulin results in an enrichment of elongated cells with a short mitotic spindle, which is indicative of a cell cycle arrest at metaphase. Thus, the GFP-α-tubulin strain can be used to perform the screen outlined in Specific Aim 3, and the use of this strain is an improvement to my original proposal which will enable me to screen more mammalian cDNAs to determine if they induce a metaphase arrest in *S. pombe*. However, I have spent more time than anticipated performing the tasks in Specific Aim 1. These tasks have been productive and have led to the identification of five novel alleles of known spindle checkpoint genes, as detailed in this report. For this reason, I have not yet completed the tasks outlined in Specific Aim 3.

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